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Biotransformation of *p*-, *m*-, and *o*-hydroxybenzoic acids by *Panax ginseng* hairy root cultures

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Abstract

The regioselective glycosylation of three isomers of hydroxybenzoic acids was observed in *Panax ginseng* hairy root cultures. *p*-Hydroxybenzoic acid (**1**) and *m*-hydroxybenzoic acid (**2**) were converted into their corresponding glycosides (**1a** and **2a**) and glycosyl esters (**1b** and **2b**) while no metabolite of *o*-hydroxybenzoic acid (**3**) was detected. A new compound, *m*-hydroxybenzoic acid β-D-xylopyranosyl (1 → 6)-β-D-glycopyranosyl ester (**2c**) was identified as a biotransformation product of **2**. Further time-course studies of the biotransformation reactions showed that the glycosides were major products in the latter stage. The addition of carbohydrates or antioxidants increased glycosyl esters formation.

Keywords: Biotransformation; Glycosylation; Hydroxybenzoic acid; *Panax ginseng*; Hairy root cultures

1. Introduction

Glycosylation reactions are of special interest because they facilitate the conversion of water-insoluble compounds to those that are more water-soluble [1]. A great number of glycosylation studies using plant cell cultures or hairy root cultures have been carried out [2–16] since such reactions are very difficult by microbial transformations or by chemical means [1].

Panax ginseng cell and hairy root cultures are widely used and versatile biotransformation tools. Such cultures enzymatically transform phenolics [6,17–20], coumarin [21], digitoxigenin [22,23] and 18β-glycyrrhetinic acid [12] to their corresponding glycosides. In our previous studies, we reported the glycosylation of phenolic hydroxyl groups on hydroquinone [24,25] and *p*-hydroxybenzyl alcohol [26] by *Panax ginseng* hairy roots induced from ginseng tender stems [27]. *p*-Hydroxybenzyl alcohol was efficiently transformed to gastrodin, the active component of the famous anti-headache Chinese herb, *Gastrodia elata* Bl.

As a continuation of our work on the specificities and selectivities of *Panax ginseng* hairy root culture biotransformations of phenolic hydroxyl and carboxyl groups, *o*-, *m*- and *p*-hydroxybenzoic acids were undertaken. These substrates were chosen because (1) it is possible to assess the regioselectivity and the group priority selection of the glycosylation reaction for phenolic hydroxyl and carboxylic acid groups within a single structure; (2) these substrates are building blocks of some complex active natural products such as rhein, pseudopurpurin, and sennidin; and (3) the glycosylation of such phenolic benzoates may reveal novel characteristics of glycosylation reactions.

2. Material and methods

2.1. Instruments and general methodology

HPLC analyses were performed using an Agilent 1100 instrument equipped with a HP chemstation, model G1312A binary pump, model G1313A micro autosampler, model G1316A thermostated column compartment, and a model G1314A variable wavelength detector. ESI-LC/MS was obtained using an Agilent 1100 LC-MSD series trap. The column was a 4.6 mm × 250 mm, 5 μm, Alltima C_{18} analytical column (Alltech Associates, Inc., USA) maintained at 25 °C equipped with a 3.0 mm × 7.5 mm C_{18} precolumn. ^{1}H and ^{13}C NMR spectra were measured with

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a Bruker Avance 400 spectrometer in methanol-d_4 solution with TMS as the internal reference, and chemical shifts are expressed in δ (ppm). Thin layer chromatography (TLC) was conducted using silica gel GF_{254} plates purchased from Qingdao Marine Chemical Group, China. All of the substrates were purchased from Shanghai Chemical Co. Ltd., and the purities were above 98% as determined by HPLC analyses.

Compounds **1**, **2** and their metabolites were analyzed by HPLC using 5% acetonitrile and 95% water (containing 1% formic acid) as solvent at a flow rate of 1.0 ml/min with UV detection at 242 nm. Retention times (min) under these conditions were as follows: **1a**, 9.2; **1b**, 15.1; **1**, 30.5; **2a**, 16.3; **2b**, 24.9; **2c**, 37.8; **2**, 45.6.

2.2. Plant materials and culture methods

Panax ginseng hairy root cultures were subcultured as previously described on B_5 medium supplemented with 2% (w/v) sucrose and 0.5 g/l lactalbumin hydrolysate on a rotary shaker (100 rpm) at 25 °C in the dark [24].

2.3. Biotransformation of p-, m- and o-hydroxybenzoic acids by Panax ginseng hairy root cultures

The substrate (20 mg) was dissolved in 0.5 ml ethanol and added to a 75 ml suspension of hairy roots pre-cultured for 4 weeks in a 150 ml Erlenmeyer flask. Controls received only 0.5 ml of the vehicle, ethanol. Following 7 days of incubation, the cultures were filtered, the roots were lyophilized and then extracted by boiling with methanol at 68 °C for 2 h. The methanol extract was concentrated to 10 ml in a rotary evaporator. The culture filtrate was extracted with equal volumes of *n*-butyl alcohol for four times, and the extract concentrated by evaporation in vacuo to a residue that was dissolved with 10 ml methanol. Extracts from roots and filtrate were examined by HPLC.

Structures of products were established by ESI-LC/MS and 2D NMR spectroscopic techniques. ^{13}C NMR spectral data of glycosylation products are shown in Table 1.

2.4. Administered additional carbohydrates and antioxidants

Addition of 1.5 g of various carbohydrates (sucrose, glucose, lactose or mannitol) or 20 mg of antioxidants (gallic acid or ascorbic acid) were made to hairy root cultures together with **2**. Incubations were continued for another 7 days. For this experiment, the control used only **2** without any additional compounds.

3. Results and discussion

3.1. Biotransformation of p-hydroxybenzoic acid

Addition of **1** to *Panax ginseng* hairy root cultures gave two compounds, which were found in both roots and the medium but not in the control. Purification by C_{18} column chromatography and preparative HPLC gave products **1a** and **1b** in 17.7 and 14.9% yields, respectively. Their structures were respectively identified as *p*-carboxyphenyl β-D-glycopyranoside (**1a**) and *p*-hydroxybenzoic acid β-D-glycopyranosyl ester (**1b**) according to the MS, ^1H NMR, ^{13}C NMR, DEPT, HSQC and HMBC spectra analysis (Fig. 1).

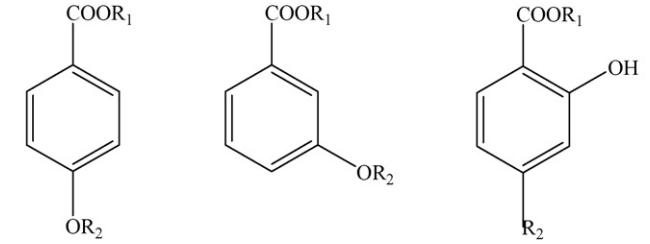
Table 1
^{13}C NMR data of for **1a**, **1b**, **2a**, **2b**, **2c** obtained by biotransformations of hydroxybenzoic acids by hairy root cultures of *Panax ginseng*

C	1a	1b	2a	2b	2c
1	120.1	120.2	123.3	120.6	121.3
2	131.6	132.4	132.1	130.6	130.8
3	116.3	115.8	157.7	157.5	158.0
4	161.3	162.9	120.9	120.3	120.7
5	116.3	115.8	117.5	116.0	116.4
6	131.6	132.4	129.1	129.2	130.3
7	167.4	164.9	168.2	165.4	165.0
1′	100.2	95.0	100.9	94.8	95.3
2′	73.6	73.0	73.4	72.7	72.9
3′	77.6	78.4	76.8	77.5	77.0
4′	70.0	70.0	69.8	69.7	69.9
5′	77.0	76.9	76.5	76.7	76.6
6′	61.0	61.0	61.0	60.9	68.5
1″					104.2
2″					73.7
3″					77.0
4″					69.7
5″					66.1

A time-course study of the bioconversion of **1** to products showed that **1** was rapidly converted into **1a** and **1b** after 6 h (Fig. 2). Substrate **1** was rapidly consumed during the first 12 h, a plateau was reached for the next 12 h and substrate consumption continued for another day. The yield of both products gradually increased over the initial 5-day incubation with higher levels of **1b** initially, and roughly equal yields at about 15% for each compound after 6 days.

3.2. Biotransformation of m-hydroxybenzoic acid

Panax ginseng hairy root cultures converted **2** into three metabolites designated as **2a**, **2b**, and **2c**, which were purified by C_{18} column chromatography and preparative HPLC. Both **2a** and **2c** were found in the roots whereas **2b** was detected in both the medium and roots. None of them was



1: R_1=R_2=H
1a: R_1=H, R_2=Glc
1b: R_1=Glc, R_2=H

2: R_1=R_2=H
2a: R_1=H, R_2=Glc
2b: R_1=Glc, R_2=H
2c: R_1=Glc(6-1)Xyl, R_2=H

3: R_1=R_2=H
4: R_1=Na, R_2=H
5: R_1=H, R_2=SO_3H

Fig. 1. Structures of substrates and their glycosylation products.

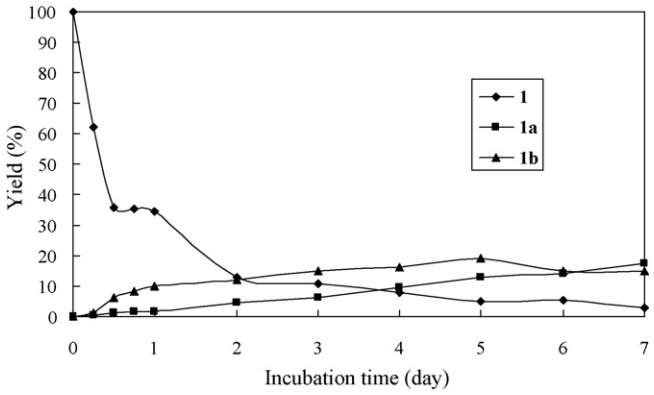


Fig. 2. Time-course in the biotransformation of **1** by *Panax ginseng* hairy root cultures. Yields are expressed as a percentage relative to the total amount of reaction products on a molar basis.

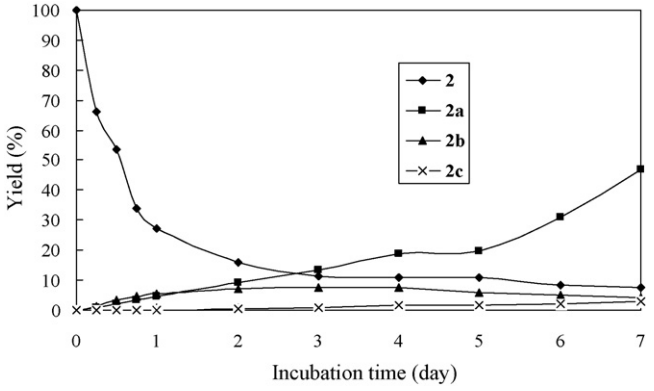


Fig. 3. Time-course in the biotransformation of **2** by *Panax ginseng* hairy root cultures. Yields are expressed as a percentage relative to the total amount of reaction products on a molar basis.

found in the control. Their structures were identified as *m*-carboxyphenyl β-D-glycopyranoside (**2a**), *m*-hydroxybenzoic acid β-D-glycopyranosyl ester (**2b**), and *m*-hydroxybenzoic acid β-D-xylopyranosyl (1 → 6)-β-D-glycopyranosyl ester (**2c**) utilizing MS, ^{1}H NMR, ^{13}C NMR, DEPT, HSQC and HMBC analysis (Fig. 1). Notably, **2c** was a new compound, the physical properties of which follow. ^{13}C NMR spectral data are shown separately in Table 1.

m-Hydroxybenzonic acid β-D-*xylopyranosyl* (1 → 6)-β-D-*glycopyranosyl ester* (**2c**): white powder; $C_{18}H_{24}O_{12}$; mp 138–141 °C; ESI-MS *m/z* 431 $[M–H]^-$, 293 $[M–C_7H_5O_3–H]^-$, 149 $[C_5H_{10}O_5–H]^-$, 137 $[C_7H_5O_3]^-$. ^{1}H NMR (DMSO-d_6) δ 7.45 (d, 1H, *J* = 7.8, H-6), 7.41 (s, 1H, H-2), 7.34 (t, 1H, *J* = 7.8, H-5), 7.06 (dd, 1H, *J* = 7.9, 2.3, H-4), 5.55 (d, 1H, *J* = 7.5, H-1′), 4.13 (d, 1H, *J* = 7.5, H-1″), 3.94 (dd, 1H, *J* = 10.7, 1.9, H-6′a), 3.57 (dd, 1H, *J* = 11.0, 5.6, H-6′b), 3.66 (dd, 1H, *J* = 11.4, 5.5, H-5″a), 3.06 (dd, 1H, *J* = 11.4, 8.6, H-5″b), 2.95–3.47 (*m*, 7H, H-2′,3′,4′,5′,2″,3″,4″).

The ESI-MS spectrum of **2c** displayed an $[M–H]^-$ ion peak at *m/z* 431, indicating a molecular formula of $C_{18}H_{24}O_{12}$. Distinct from that of **2**, additional pentose and hexose units were conjoined to the compound. According to the ^{1}H and ^{13}C NMR spectra (Table 1), the chemical shifts and the coupling constants of the sugar moiety of **2c** were in good agreement with those of **2** with primeverose. The ESI-MS spectral fragments at *m/z* 293 and *m/z* 149 also indicated the presence of primeverose and a pentose. The sugar attachment at the carboxyl group of **2c** was β as indicated by the signals assigned to the anomeric proton and carbon of the inner glucose moiety at δ5.55 (d, 1H, *J* = 7.5, H-1) and 95.3 (C-1). Thus **2c** was the β-D-primeverose ester of **2**. All the data indicated that **2c** was *m*-hydroxybenzoic acid β-D-xylopyranosyl (1 → 6)-β-D-glycopyranosyl ester.

A time-course biotransformation study showed that **2** was rapidly converted into **2a** and **2b** starting at 6-h incubation, with only traces of **2c** detected after 1 day (Fig. 3). Glycosyl ester (**2b**) and glycoside (**2a**) yields were similar early in the biotransformation reaction with **2a** becoming the major product after a 2-day reaching a maximum yield of 47% after 7 days.

It is noticed that although *Panax ginseng* hairy root cultures can glycosylate either hydroxyl or carboxyl groups of **1** and **2**, hydroxyl group glycosylations gave higher yields.

3.3. Investigation of biotransformation of o-hydroxbenzoic acid and its analogs

No biotransformation products were obtained with **3** as substrate. Inspired by previous findings that methyl salicylate was glycosylated by *Panax ginseng* root cultures [19], we administered analogs of **3**, such as sodium salicylate (**4**) and sulphosalicylic acid (**5**). Neither of which gave biotransformation products. The results indicated that ortho-hydroxy benzoic acids were not glycosylated by *Panax ginseng* root cultures.

3.4. The influence of carbohydrate and antioxidant additions on glycosylation

Additions of carbohydrates and antioxidants to cell cultures may enhance biotransformation conversion rates [28]. As shown in Fig. 4, all four added carbohydrates increased the overall conversion of **2** to **2a**, **2b** and **2c**, and especially **2c** versus controls. Among them sucrose gave the most significant improvement and the total production amounts increased 34% compared with the control. At the same time, mannitol, lactose and glucose also

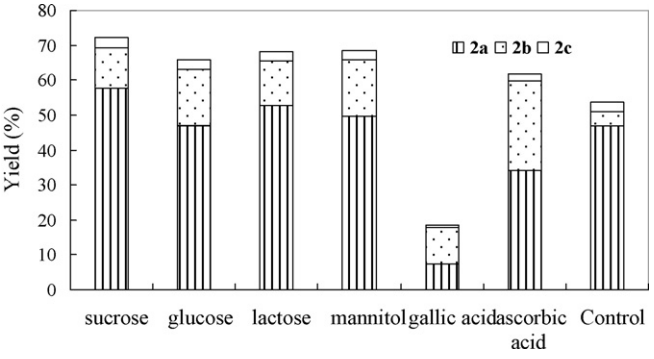


Fig. 4. Influence of additions of carbohydrates and antioxidants on glycosylations of **2**. Each column shows the corresponding yield of product **2a**, **2b** and **2c**.

improved glycosylation by the 27, 26 and 22%, respectively. Ascorbic acid addition slightly increased the conversion reaction by about 14% while gallic acid addition reduced the glycosylations of **2** by 66%. Among the three glycosylation products of **2**, **2b** yields were most dramatically influenced by all of the additives. Ascorbic acid increased **2b** yield 6.3-fold while mannitol, glucose, lactose, sucrose and gallic acid enhanced **2b** yields by 4, 4, 3, 3, 2.6 times, respectively. Thus, it appears that additive carbohydrates and antioxidants increased the tendency for glycosylation towards the carboxylic acid moiety more prominently than towards the phenolic hydroxyl group.

4. Conclusions

Hydroxybenzoic acids have received considerable attention because of their applications in food, medicine, cosmetics and polymer industries. Phenolic compounds conjugated with glucose or other sugars are found as glycosides, widespread in higher plants where they serve various physiological functions [29–31]. *Panax ginseng* hairy root cultures were regioselective in glycosylation of two *meta*- and *para*-hydroxybenzoic acids. *Panax ginseng* transforms other phenolic compounds into their primeverosides [19,21], and a primeverosyl ester of 2-phenylpropionic acid was obtained by Furuya and co-workers [17,20]. However, the attachment of primeverose to a phenolic carboxyl group by *Panax ginseng* is the first found. Moreover, it was interesting to show that additive carbohydrates and antioxidants can enhance glycosyltransferases preference for carboxyl groups versus hydroxyl groups on substrates like **2**.

In summary, the unique catalytic capability of *Panax ginseng* hairy root cultures to glycosylations of hydroxybenzoic acids deserves further exploration. Such studies could provide new platforms for combinatorial synthesis [32] and the development of new, active phenolic compounds.

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